Allelic Loss at *Drosophila* Patched Gene Is Highly Prevalent in Basal and Squamous Cell Carcinomas of the Skin

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The human homolog of the *Drosophila* Patched gene (*PTCH*), located at chromosome 9q22.3, is frequently altered in both nevoid basal cell carcinoma syndrome, and sporadic basal cell carcinomas (BCCs). However, alteration of the *PTCH* gene locus has been poorly studied in squamous cell carcinoma (SCC). We analyzed loss of heterozygosity (LOH) at five markers in and around the *PTCH* gene in 276 keratinocyte tumors from a population-based study in New Hampshire. We found a high prevalence of any 9q22.3 LOH in both BCC (75.5%) and SCC (60.8%), with BCC being significantly more likely to have LOH than SCC ($P<0.009$). The *PTCH* gene was specifically lost in 60% of BCC, and 50% of SCC tumors. Among SCC tumors, 9q22 LOH was significantly more likely to occur in those who tend to burn ($P<0.05$), and this association was strongest for tumors that occurred on sun-exposed areas of the body ($P<0.04$). Additionally, 9q22 LOH occurred more frequently in SCC tumors associated with a history of severe sunburns ($P<0.08$). Thus, in our large, population-based sample, 9q22 loss, including *PTCH*, was highly prevalent in both BCC and SCC. Overall, these data support the hypothesis that *PTCH* loss is a common, early lesion for SCC and BCC.

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INTRODUCTION

Basal cell (BCCs) and squamous cell carcinomas (SCCs) are the most common cancers among US Caucasians (ACS, 2002), and incidence is increasing at both low and high altitudes (Karagas *et al.*, 1999; Harris *et al.*, 2001). Risk factors for these cancers include fair skin, excess sun exposure, immunosuppression, and exposure to other carcinogens such as arsenic and polyaromatic hydrocarbons (Scotto *et al.*, 1996). BCCs are the more common type of skin cancer, and are characterized by slow growth, local invasion, and rare metastasis (Preston and Stern, 1992; Quinn *et al.*, 1994a). In contrast, SCCs have a greater metastatic potential and are responsible for the majority of non-melanoma skin cancer deaths (Preston and Stern, 1992).

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Abbreviations: BCC, basal cell carcinoma; LOH, loss of heterozygosity; SCC, squamous cell carcinoma; Shh, sonic hedgehog

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BCCs most often occur as sporadic tumors, but they also develop in patients with the rare autosomal dominant disorder nevoid basal cell carcinoma syndrome (Gorlin, 1995). The gene for this disorder has been cloned and identified as the human homologue of the drosophila segment polarity gene patched (*PTCH*) located on chromosome 9q22.3 (Gailani *et al.*, 1992; Hahn *et al.*, 1996; Johnson *et al.*, 1996). As with nevoid basal cell carcinoma syndrome tumors, *PTCH* is frequently altered in sporadic BCCs (reviewed in Bale and Yu, 2001), and these data support the hypothesis that *PTCH* is a gatekeeper gene for the development of BCC (Sidransky, 1996).

PTCH encodes a transmembrane protein that serves as the receptor for sonic hedgehog (Shh) (Marigo *et al.*, 1996; Stone *et al.*, 1996). Ptch represses hedgehog target gene expression through its interaction with Smoothened (Smo), and this repression is relieved when Shh binds Ptch, or after *PTCH* has been inactivated through mutation. Ptch regulates its own expression through a negative feedback loop and inactivating mutations in *PTCH* lead to overexpression of mutant *PTCH* mRNA in BCC tumors (reviewed in Bale and Yu, 2001). Further, multiple genes in the SHH pathway have been demonstrated to be inactivated in BCC (Reifenberger *et al.*, 1998).

Although *PTCH* loss clearly contributes to the genesis of BCC, there is no established role for *PTCH* alteration in SCC. Unlike BCC tumors, overexpression of *PTCH* mRNA is not a common event in SCC or extracutaneous tumors (Eklund

et al., 1998; Zhilan *et al.*, 2003). LOH at chromosome 9q22.3 has been observed, but with imprecise estimates of the prevalence of loss (Table 4). In total, 143 SCC tumors have been investigated for LOH; the largest study contained 49 SCCs. These small sample sizes, from ethnically and geographically diverse populations, have resulted in a wide range of estimates for 9q loss (0–70%). Similarly, mutation in SCC has been evaluated in only 64 tumors (30 in one study), with mutation prevalence ranging from 0 to 15%.

PTCH alteration is a critical determinant of BCC. However, small studies of SCC have limited interpretability, and an expanded investigation including population-based SCC tumors is important in order to distinguish whether this gene is critical to both BCC and SCC. Herein, we have performed loss of heterozygosity (LOH) analysis on tumors from an ongoing large epidemiologic study of keratinocyte cancers in New Hampshire, to test the hypothesis that LOH events at *PTCH* are histology specific.

RESULTS

A total of 276 tumors from 250 cases were informative for loss using five markers on chromosome 9q22. Overall, there was a high prevalence of any LOH on 9q22 in both SCC (60.8%) and BCC (75.5%) (Table 1, Figure 1). BCC tumors were 2 times more likely than SCC to have any loss on 9q22.3 ($P<0.009$). The *PTCH* gene was specifically lost in 50% of SCC and 60% of BCC tumors. To estimate whether the extent of LOH differed by histology, we calculated fractional allele loss (number of lost markers/number of informative markers) for each tumor, and there was no difference in this measure by histology (data not shown). In addition, the prevalence of LOH did not differ according to the amount of adjacent normal tissue (68% LOH in the quartile with the fewest tumor cells, and 62% LOH among tumors with less than 25% normal tissue).

BCC and SCC were evaluated separately to identify epidemiologic determinates of *PTCH* LOH. For each variable, a χ^2 or Wilcoxon's rank-sum test was used to test whether there was a significant difference in the prevalence of LOH with exposures or traits (Table 2). For BCC, the prevalence of LOH was constant across skin type groups. In addition, there were no differences in the number of lifetime sunburns for those with and without LOH, nor was histologic solar elastosis, a measure of chronic photoexposure, associated

Table 1. 9q22.3 LOH in BCC and SCC of the skin

Marker	Chromosome location[1]	BCC	SCC	*P*-value[2]
D9S15	69571447–69571598			
Retain		67 (81.7%)	64 (81%)	
LOH present		15 (18.3%)	15 (19%)	0.9
D9S196	93553591–93553855			
Retain		17 (27.8%)	24 (46.1%)	
LOH present		42 (72.2%)	28 (53.9%)	0.06
Ptch exon 1a[3]	95287716–95287944			
Retain		24 (40%)	19 (50%)	
LOH present		36 (60%)	19 (50%)	0.3
D9S176	99137859–99138032			
Retain		57 (50.4%)	45 (57%)	
LOH present		56 (49.6%)	34 (43%)	0.4
D9S53	104641192–104641317			
Retain		59 (61.5%)	57 (74%)	
LOH present		37 (38.5%)	20 (26%)	0.08
Any LOH[4]				
Absent		37 (24.5%)	49 (39.2%)	
Present		114 (75.5%)	76 (60.8%)	0.009

BCC, basal cell carcinoma; LOH, loss of heterozygosity; SCC, squamous cell carcinoma.
[1]bp location according to NCBI UniSTS database, for exon1a the gene start position is given.
[2]χ^2 test of independence for marker loss and tissue histology.
[3]Dinucleotide repeat IAJL (Louhelainen *et al.*, 1998).
[4]Among those informative for at least one marker.

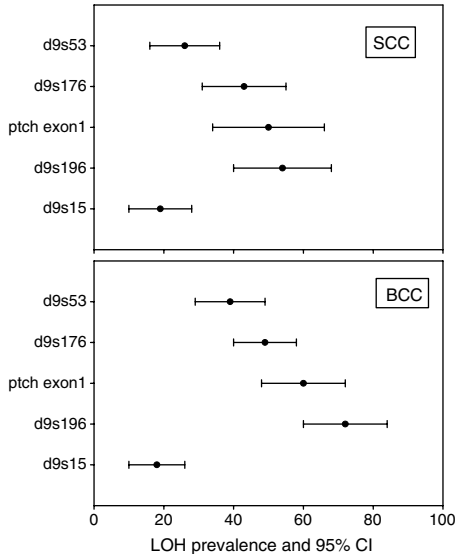

Figure 1. Prevalence and 95% confidence interval for LOH at five markers in SCC and BCC tumors.

with LOH. Considering these same variables and SCC (Table 2), there were statistically significant differences in LOH by skin type, with the prevalence of LOH significantly lower among those who always tan ($P<0.05$), and a non-significant trend for increased LOH with lifetime sunburns ($P<0.08$).

Tumors were stratified by anatomic site, broadly grouped into sun-exposed (head, neck, arms) and sun-protected (trunk and legs). We tested whether the initial observation of increased LOH in BCC *versus* SCC (Table 1) was similar among anatomic site groupings (Figure 2). The strong difference in LOH by histology ($P<0.009$, Table 1 and Figure 2) was only evident on sun-protected but not sun-exposed sites ($P<0.002$ *vs* $P<0.2$, Figure 2). We further examined whether epidemiologic determinants of LOH differed by anatomic site of the tumor (Table 3). All BCC tumors on sun-protected sites had 9q22 LOH among those who reported sunlamp use, compared to 76.7% among non-users of sunlamps, and this difference was of borderline statistical significance ($P=0.05$). In addition, for SCC, the association between increased LOH and tendency to sunburn was only evident for tumors on sun-exposed areas of the body ($P<0.04$).

DISCUSSION

Our data demonstrate that LOH in the *PTCH* region on 9q22 is a highly prevalent event in both BCC and SCC, with BCC tumors having significantly more deletion events in the *PTCH* region than SCC. Loss was highest in and around the *PTCH* gene, suggesting that the gene itself is targeted for alteration in both BCC and SCC. In addition, the detection of LOH was not sensitive to the amount of pathologically normal tissue, indicating that the tumor is homogeneous for LOH. The frequency of loss observed for BCC is comparable to what has been reported previously for tumors from sporadic cases (Gailani *et al.*, 1992; Quinn *et al.*, 1995; Hahn *et al.*, 1996; Holmberg *et al.*, 1996; Johnson *et al.*, 1996; Shen *et al.*, 1999; Ling *et al.*, 2001; Kim *et al.*, 2002; Asplund *et al.*, 2005; Reifenberger *et al.*, 2005). Prior studies of SCC have reported a wide range of 9q22 LOH prevalence (0–70%), likely due to the small number of tumors studied in any given report (reviewed in Table 4). Here, in a large case series, we found that 9q markers, including a *PTCH* -specific marker, are lost in a high proportion of SCC (60%). Small sample sizes can lead to imprecise estimates of true population prevalence, and this may partly explain why our results differ from those of the previous reports. In addition, our study sampled from all treating physicians within a geographically defined region, and our larger sample size permitted us to examine tumors from sun-exposed and sun-protected sites separately. It is possible that prior reports represented populations with different traits and exposures than our New Hampshire population (ie sun sensitivity), that relate to the prevalence of LOH in SCC reported here. In addition, by not relying on a tertiary care center for the collection of cases we have not biased our sample by the clinical phenotype of the tumors (ie aggressive or invasive), which could also impact the prevalence of gene deletion.

The epidemiologic design of our study allowed us to examine how patient traits and exposures relate to 9q22 loss. In BCC, LOH occurred independent of age, gender, skin type, and sunburn history. However, for SCC, *PTCH* region deletion was highest among those that burn and had a higher number of lifetime sunburns. In contrast, the occurrence of 9q22 LOH did not vary by measures of chronic actinic exposure (ie extent of solar elastosis). This suggests that indicators of acute, intense UV exposure, as occurs with sunburning, may lead to 9q22 LOH in SCC tumors. Sunburning also is consistent with the etiology of BCC, and these data suggest that a subset of SCC may have a common origin to BCC both in terms of exposure and early molecular alterations.

Our observation of significant loss of 9q, including the *PTCH* gene region, in SCC tumors suggests that 9q or the *PTCH* gene itself is crucial to SCC development. This is supported by *in vitro* work as mice heterozygous at the *PTCH* locus develop SCC tumors following UV exposure (Aszterbaum *et al.*, 1999), as well as bladder carcinomas in response to nitrosamine exposure (Hamed *et al.*, 2004). However, nevoid basal cell carcinoma syndrome patients are not reported to develop excess SCC tumors. As chromosome 9q22 is dense with documented tumor suppressor genes, including *FANCC* and *XPA*, it is possible that it is one of these adjacent genes that are targeted for loss in SCC. Additional research, possibly using fine mapping techniques, is needed to resolve the question of deletions at 9q22 in SCC.

In BCC disease, it has been well demonstrated that mutations in *PTCH* lead to overexpression of ptch mRNA, and that two gene inactivation events normally occur. The balance between *PTCH*, *Shh*, and *Smo* expression is essential for normal signaling, and any disruption (such as LOH) of

Table 2. Epidemiologic determinants of LOH in the *ptch* gene region

	BCC			SCC		
	9q retained	**9q LOH**	***P*-value**	**9q retained**	**9q LOH**	***P*-value**
Sex						
Male	28 (28.3%)	71 (71.7%)		34 (38.6%)	54 (61.4%)	
Female	9 (17.3%)	43 (82.7%)	0.1	15 (40.5%)	22 (59.5%)	0.8
Median age (years)	64	62		68	67	
(range)	(32 – 74)	(29 – 73)	0.6	(39–74)	(39–74)	0.7
Skin type[1]						
Tans	4 (28.6%)	10 (71.4%)		10 (62.5%)	6 (37.5%)	
Burns then tans	16 (23.2%)	53 (76.8%)		17 (29.8%)	40 (70.2%)	
Severe burn	17 (25%)	51 (75.0%)	0.9	22 (43.1%)	29 (56.9%)	0.05
Painful sunburns[2]						
0	11 (35.5%)	20 (64.5%)		20 (56.7%)	17 (43.3%)	
1–3	6 (15%)	34 (85%)		12 (32.4%)	25 (67.6%)	
4+	20 (25%)	60 (75%)	0.1	16 (32.6%)	33 (67.4%)	0.08
Sunlamp use						
No	32 (27.8%)	83 (72.2%)		43 (41.7%)	60 (58.3%)	
Yes	5 (13.9%)	31 (86.1%)	0.09	6 (27.3%)	16 (72.7%)	0.2
Histologic solar elastosis						
Minimal	1 (7.7%)	12 (92.3%)		0	1 (100%)	
Moderate	11 (25.6%)	32 (74.4%)		6 (40%)	9 (60%)	
Severe	16 (26.2%)	45 (73.8%)	0.3	38 (39.2%)	59 (60.8%)	0.7

BCC, basal cell carcinoma; LOH, loss of heterozygosity; SCC, squamous cell carcinoma.
[1]Skin response to first sun exposure in summer.
[2]Lifetime number of sunburns causing pain for 2 or more days.

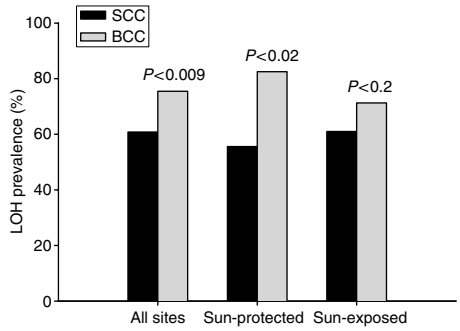

Figure 2. The prevalence of any 9q22 LOH in SCC and BCC, stratified by anatomic site of the tumor.

PTCH could lead to tumor formation. In the case of SCC, it is plausible that there is no second mutation event at *PTCH*. A similar scenario has been observed for bladder cancers, where 9q22.3 appears targeted for genetic deletion (Cairns *et al.*, 1993; Simoneau *et al.*, 1999, 2000; Kimura *et al.*, 2001; Wada *et al.*, 2003), with little evidence for *PTCH* mutation (Xie *et al.*, 1997; McGarvey *et al.*, 1998; Ohgaki *et al.*, 1999; Aboulkassim *et al.*, 2003). Haploinsufficiency at the *PTCH* locus has been proposed as contributing to bladder carcinogenesis (Aboulkassim *et al.*, 2003) and medulloblastomas (Wetmore *et al.*, 2000; Zurawel *et al.*, 2000), a model that may also apply to SCC (Aszterbaum *et al.*, 1999). Recently, it has been reported that uniparental disomy is a common mechanism of *PTCH* gene inactivation in BCC (Teh *et al.*, 2005). This is consistent with a two-hit mechanism for ptch in BCC, with LOH occurring without a change in genomic copy number. If this mechanism is unique to BCC, and SCC tumors retain only a single 9q allele, it could partially explain the lack of excess ptch RNA in SCC tumors. In addition, BCC tumors from ptch heterozygote animals have enhanced ptch promoter activity, whereas SCC tumors do not (Aszterbaum *et al.*, 1999), indicating that the feedback loops for these two types of tumors substantially differ.

Table 3. Anatomic site of tumor modifies the prevalence of LOH

	Sun-exposed		Sun-protected	
	BCC	SCC	BCC	SCC
Skin type				
Tans	7/10 (70%)	5/15 (33.3%)	3/4 (75%)	1/1 (100%)
Burns then tans	30/45 (66.7%)	37/53 (69.8%)	23/24 (95.8%)	2/3 (66.7%)
Severe burn	30/39 (76.9%)	22/37 (59.5%)	21/29 (72.4%)	6/13 (46.2%)
P-value	0.6	0.04	0.08	0.5
Painful sunburns				
0	13/20 (65%)	15/30 (50%)	7/11 (63.6%)	1/6 (16.7%)
1–3	22/27 (81.5%)	21/32 (65.6%)	12/13 (92.3%)	4/5 (80%)
4+	32/47 (68.1%)	28/42 (66.7%)	28/33 (84.9%)	4/6 (66.7%)
P-value	0.4	0.3	0.2	0.08
Sunlamp use				
No	50/72 (69.4%)	52/90 (57.8%)	33/43 (76.7%)	7/12 (58.3%)
Yes	17/22 (77.3%)	12/15 (80%)	14/14 (100%)	3/6 (50%)
P-value	0.5	0.1	0.05	0.7
Histologic solar elastosis				
Minimal	1/1 (100%)	1/1 (100%)	11/12 (91.7%)	Not observed
Moderate	14/20 (70%)	6/10 (60%)	18/23 (78.3%)	3/5 (60%)
Severe	37/50 (74%)	53/85 (62.4%)	8/11 (72.7%)	4/10 (40%)
P-value	0.8	0.7	0.5	0.5

BCC, basal cell carcinoma; LOH, loss of heterozygosity; SCC, squamous cell carcinoma.

Table 4. 9q22 and *PTCH* alterations in cutaneous SCC

Reference	Sample size	9q22 LOH (%)	*PTCH* mutation (%)	*PTCH* expression
Current study, Danaee *et al.* (2005)	130	62		
Granga *et al.* (2003)	30		0	
Ping *et al.* (2001)	20		15	
Mortier *et al.* (2000)	27	70		
He *et al.* (1999)[1]	21	0		
Nagano *et al.* (1999)	8			50% (weak signal, IHC)
Eklund *et al.* (1998)	14	50	0	0% (*n*=6, RNA)
Ahmadian *et al.* (1998)	11	63		
Holmberg *et al* (1996)	11	25		
Quinn *et al.* (1994b)	49	33		
Zaphiropoulos *et al.* (1994)	10	40		

[1]Only one marker examined.
IHC, immunohistochemistry; LOH, loss of heterozygosity; SCC, squamous cell carcinoma.

The data from this population-based study indicate that *PTCH* alteration is not specific for BCC, and that loss of one allele is common to both SCC and BCC. It is plausible that SHH pathway deregulation is a necessary event for dysregulated skin growths, as a high prevalence of *PTCH* alteration, including LOH, has also been observed in trichoepitheliomas (Vorechovsky *et al.*, 1997; Matt *et al.*, 2000) and sebaceous nevi (Xin *et al.*, 1999). Further characterization of the *PTCH*

gene and *Shh* pathway in epithelial malignancies is needed and may help illuminate the mechanism of histologic divergence in keratinocyte cancers.

MATERIALS AND METHODS

Study population

Cases were selected from a statewide incidence survey of dermatologists and pathology laboratories that identifies newly diagnosed primary BCC and SCC in New Hampshire, in operation since 1993 (Karagas *et al.*, 1999). For the parent study, all cases of invasive SCC, and a randomly selected group of BCC (roughly in a 1:1 ratio to SCC cases) diagnosed between July 1, 1997 and March 31, 2000 were invited to participate in a detailed epidemiologic study. A comprehensive pathology review was conducted by the study dermatopathologist, including classification of tumor morphology, grade, associated actinic keratoses, degree of histologic solar elastosis, and percent of tumor present in the tissue specimen.

Cases completed an administered questionnaire to obtain information on demographic traits. Exposure variables included in the current analysis are as follows: (1) skin type, classified according to the participant's recollection of skin response to first sun exposure at the beginning of summer, (2) number of lifetime painful sunburns (painful for 2 or more days), and (3) anatomic site of tumor classified as sun-exposed (head, neck, arms) or sun-protected (trunk and legs).

A subset of cases was analyzed in the current study. Two hundred and eighty-two tumors from 262 cases were included in the current analysis. Six cases were not informative at any of the tested loci, leaving a total of 276 tumors from 250 cases. For 16 cases, we investigated multiple tumors (ranging from 2 to 5).

Each tumor was scored by the study pathologist regarding the amount of non-tumorous tissue present in the specimen (0–25, 25–50, 50–75, >75%), and LOH was subsequently evaluated in each of these categories to ensure results were not biased by the presence of non-tumorous tissue.

All elements of the research design were approved by the Dartmouth Medical School and Harvard School of Public Health Institutional Review Boards, and informed consent was provided by all study participants. The study was conducted according to the Declaration of Helsinki Principles.

LOH analysis

Tumor DNA was extracted from diagnostic specimens obtained from the treating pathologist or pathology laboratory. Three 20-μm sections from the paraffin block were cut and placed in an Eppendorf tube. Paraffin was dissolved using Histochoice Clearing Agent (Sigma-Aldrich, St Louis, MO), followed by two washes with 100% ethanol and one wash with phosphate-buffered saline. Samples were incubated overnight in SDS-lysis solution and proteinase K (Qiagen, Valencia, CA), at 55°C. De-crosslinking was achieved by incubation with NaCl at a final concentration of 0.7 M, and DNA recovered using the Wizard DNA clean-up kit (Promega, Madison, WI). Corresponding peripheral blood DNA was isolated for each case (Qiagen).

Five microsatellite loci, D9S15, D9S53, D9S196, D9S176, including an intragenic microsatellite in *PTCH* (exon 1a, *1AJL*; Louhelainen *et al.*, 1998) were examined. Fluorescent dye labeled primer pairs for each locus were obtained from Research Genetic's Map Pair list (Invitrogen, Carlsbad, CA). Each PCR mixture contained 1.5 μl of template DNA, 12.5 pmol of each primer, 0.2 mM deoxynucleoside triphosphates, 1 μl of 10× reaction buffer, and 1.25 units of AmpliTaqGold (Applied Biosystems, Foster City, CA) in a final volume of 12.5 μl. Reaction conditions were as described by Shen *et al.* (1999), with varying annealing temperatures for each loci being studied. Briefly, 40 cycles of amplification were performed: 94°C for 40 seconds, annealing at 55–64°C (depending on primer set) for 1 minute and extension at 72°C for 1 minute. A final extension was done for 10 minutes. Amplified PCR products were run on a 4% denaturing polyacrylamide gel for ABI PRISM 377 (Applied Biosystems) with a labeled marker (TAMRA 350) as an internal size standard. The data were analyzed with the Genescan 2.1 software (Applied Biosystems). For each informative locus, the allelic ratio was calculated using the peak heights from the blood and tumor-derived DNA samples. LOH was determined by dividing the allelic ratio in the blood-derived DNA by the allelic ratio in the tumor-derived DNA. Ratios of <0.5 or >1.5 were scored as positive for LOH.

Statistical analysis

Determinants of LOH were investigated using univariate (χ^2 and Wilcoxon's rank-sum test) and multivariate methods. Odds ratios were calculated using unconditional logistic regression. All statistical analyses were performed using the SAS software system and all statistical tests were two-sided.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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